Published in final edited form as:

Brain Res. 2016 July 1; 1642: 336–343. doi:10.1016/j.brainres.2016.04.014.

Surface expression of GABA_A receptors in the rat nucleus accumbens is increased in early but not late withdrawal from extended-access cocaine self-administration

Anthony Purgianto, Jessica A. Loweth, Julia J. Miao, Mike Milovanovic, and Marina E. Wolf*

Department of Neuroscience, Rosalind Franklin University of Medicine and Sciences, 3333 Green Bay Rd, North Chicago, Illinois, USA

Abstract

It is well established that cocaine-induced changes in glutamate receptor expression in the nucleus accumbens (NAc) play a significant role in animal models of cocaine addiction. Far less is known about cocaine-induced changes in GABA transmission, despite its importance in regulating NAc output via local interneurons and medium spiny neuron (MSN) axon collaterals (GABA ‘microcircuit’). Here we investigated whether GABA_A receptor surface or total expression is altered following an extended-access cocaine self-administration regimen that produces a time-dependent intensification (incubation) of cue-induced cocaine craving in association with strengthening of AMPA receptor (AMPA) transmission onto MSN. Rats self-administered cocaine or saline (control condition) 6 h/day for 10 days. NAc tissue was obtained and surface proteins biotinylated on three withdrawal days (WD) chosen to span incubation of craving and associated AMPAR plasticity: WD2, WD25 and WD48. Immunoblotting was used to measure total and surface expression of three GABA_A receptor subunits (α 1, α 2, and α 4) that are strongly expressed in the NAc. We found a transient increase in surface, but not total, expression of the α 2 subunit on WD2 from cocaine self-administration, an effect that was no longer observed by WD25. The expression of α 1 and α 4 subunits was not altered at these withdrawal times. On WD48, when AMPAR transmission is significantly potentiated, we did not find any alteration in GABA_A receptor surface or total expression. Our findings suggest that the strengthening of AMPAR-mediated glutamate transmission in the NAc is not accompanied by compensatory strengthening of GABAergic transmission through insertion of additional GABA_A receptors.

Keywords

abstinence; biotinylation; cocaine self-administration; GABA_A receptors; incubation of craving; nucleus accumbens

*Corresponding Author: Marina E. Wolf, Ph.D., Department of Neuroscience, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Rd, North Chicago, IL 60064, USA; marina.wolf@rosalindfranklin.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

The authors declare no conflicts of interest.

1. Introduction

The nucleus accumbens (NAc) is an important structure within the limbic system that modulates goal-directed behaviors including those related to drug addiction (Groenewegen et al., 1999; Kelley, 1999). The NAc itself is composed mainly (~95%) of medium spiny neurons (MSN), which are GABAergic projection neurons that send both intra-NAc axon collaterals and efferent projections to various areas outside the NAc, including the ventral mesencephalon and ventral pallidum (Meredith, 1999; Sesack & Grace, 2010). Most of the remaining neurons are GABAergic interneurons that can be divided into multiple classes based on protein expression and electrophysiological properties (Tepper et al., 2010; Silberberg & Bolam, 2015). Extensive studies have documented alterations in glutamate receptor expression on NAc MSN after cocaine exposure (Wolf & Ferrario, 2010; Wolf, 2016). However, even though glutamatergic afferents are critical in shaping MSN activity (Meredith et al., 2008), the GABA microcircuit comprised by MSN collaterals and GABA interneurons also plays a major role (Wilson, 2007; Tepper et al., 2004; Silberberg & Bolam 2015).

The importance of the GABA microcircuit for NAc function has been established through anatomical and electrophysiological studies. For example, one type of GABAergic interneuron, the parvalbumin-positive (PV+) fast spiking interneuron, has been shown to synapse with dorsal striatal MSN on proximal dendrites and perikarya, suggesting a strong modulatory effect on the MSN (Bennett & Bolam, 1994). Similarly, the connection between PV+ interneurons and MSN in the NAc has been established by both anatomical (Hussain et al., 1996) and electrophysiological studies (Taverna et al., 2007). This connection has physiological importance, as stimulation of these interneurons results in powerful inhibition of MSN (Pennartz & Kitai, 1991; Koos & Tepper, 1999; Gruber et al., 2009; Gittis et al., 2010). NAc function is also regulated by GABAergic MSN-to-MSN synaptic connectivity (Tunstall et al., 2002; Taverna et al., 2004; Koos et al., 2004).

In electrophysiological studies mentioned above, GABA_A receptors were implicated in mediating the action of GABA within the microcircuit based on the latency of inhibition and sensitivity to either picrotoxin or bicuculline (Pennartz & Kitai, 1991; Koos & Tepper, 1999; Gruber et al., 2009; Gittis et al., 2010). GABA_A receptors are pentameric chloride channels composed mainly from various isoforms of α , β , and γ subunits (Olsen & Sieghart 2008), and the subunit composition determines receptor function, pharmacology, and location (Olsen & Sieghart 2009). In the NAc, the most commonly expressed GABA_A receptor subtypes are $\alpha 2\beta\gamma 2$, $\alpha 1\beta\gamma 2$, and $\alpha 4\beta\delta$ (Pirker et al., 2000). Immunocytochemical studies have found that GABA_A $\alpha 2$ subunits are preferentially expressed on MSN while GABA_A $\alpha 1$ subunits are preferentially expressed on interneurons (Schwarzer et al., 2001; Boyes & Bolam 2007). GABA_A $\alpha 4$ subunits are located extrasynaptically on NAc MSN and are also expressed on several types of interneurons (Maguire et al., 2014). Supporting immunocytochemical studies, electrophysiological studies of MSN in the NAc have established that $\alpha 2$ -containing GABA_A receptors are present and mediate phasic inhibition of these cells (Dixon et al., 2010). Tonic inhibition of MSN through $\alpha 4$ -containing GABA_A receptors (Santhakumar et al., 2010; Maguire et al., 2014) and the presence of $\alpha 1$ -containing

GABA_A receptor-mediated currents on interneurons have also been observed (Janssen et al., 2011).

A number of studies have explored the role of GABA_A receptors in the effects of non-contingent cocaine administration in the NAc. It has been found GABA_A receptors containing the $\alpha 2$ subunit are necessary for the expression of cocaine-induced behavioral sensitization (Morris et al., 2008; Dixon et al., 2010), while repeated experimenter-administered cocaine, followed by withdrawal and a cocaine challenge, led to reduction in the expression of GABA_A $\alpha 2$ subunits in the NAc shell (Chen et al., 2007). Studies of mice with deletion of the $\alpha 4$ subunit indicate that $\alpha 4$ GABA_A receptors on D1 receptor-expressing MSN act to oppose cocaine enhancement of conditioned place preference (CPP) (Maguire et al., 2014). Fewer studies have evaluated the effects of contingent cocaine exposure on GABA_A receptor expression or function. However, available data indicate that GABA transmission in the NAc shell is differently affected by contingent and non-contingent cocaine administration (Wydra et al., 2013). This is not surprising, given substantial differences in the effects of contingent and non-contingent cocaine exposure on glutamate transmission in the NAc (Wolf & Ferrario, 2010). Interestingly, one recent study found time-dependent changes in the balance between inhibitory and excitatory synaptic transmission in the NAc shell during withdrawal from limited-access cocaine self-administration (Otaka et al., 2013).

After extended-access cocaine self-administration, cue-induced cocaine craving progressively intensifies (incubates) over the first 1–2 months of withdrawal and then remains high through at least withdrawal day (WD) 90 before declining slowly (Lu et al., 2004; Pickens et al., 2011). Expression of incubated craving after 1–3 months of withdrawal depends upon strengthening of AMPA receptor (AMPA) transmission in the NAc core through synaptic incorporation of Ca²⁺-permeable AMPARs (CP-AMPA; Conrad et al., 2008; Mameli et al., 2009; Loweth et al., 2014) as well as silent synapse formation and un-silencing (Lee et al., 2013; Ma et al., 2014). The goal of the present study is to determine if incubation of cocaine craving is also associated with alterations in GABA_A receptor levels in the NAc. We focused on three GABA_A receptor subunits that are expressed at high levels in the NAc ($\alpha 1$, $\alpha 2$, $\alpha 4$; see above) and examined three withdrawal times spanning the period over which incubation of craving and associated alterations in glutamate transmission are occurring.

2. Results

After extended-access self-administration of cocaine or saline (6 h/day for 10 days), rats were killed at 3 different withdrawal time-points. The time-points were chosen based on the development of AMPAR plasticity in NAc core during withdrawal from this regimen: withdrawal day (WD) 2 (before any changes in AMPAR subunit composition are detected), WD25 (when CP-AMPA start to accumulate), and WD48 (when stable elevation of CP-AMPA has been achieved) (Wolf & Tseng, 2012). This design was chosen so as to test the hypothesis that compensatory alterations in GABA transmission accompany changes in AMPAR transmission, as well as to encompass the period of withdrawal when cue-induced cocaine craving is progressively increasing (Lu et al., 2004). At each time-point, NAc tissue

(mainly core) from cocaine and saline self-administering rats was collected, biotinylated to selectively label surface-expressed proteins, and then analyzed by immunoblotting.

We first assessed the GABA_A α 2 subunit, which is preferentially expressed on MSN (see Introduction). We detected a significant increase in the bound fraction on WD2 in the cocaine group compared to the saline group, indicating increased surface expression of α 2-containing GABA_A receptors ($t_{(22)} = 2.48$; $*p < 0.05$) (Fig. 1b). This increase in surface expression was transient, as it was no longer detected by WD25 ($t_{(21)} = 0.17$, $p > 0.05$) (Fig. 1d) or WD48 ($t_{(19)} = 0.78$, $p > 0.05$) (Fig. 1f). There was no alteration in the total expression of the α 2 subunit at any of the three withdrawal times (WD2: $t_{(22)} = 0.81$, $p > 0.05$; WD25: $t_{(17)} = 0.01$, $p > 0.05$; WD48: $t_{(19)} = 0.48$, $p > 0.05$) (Fig. 1a,c,e). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times (surface: $F_{(1,59)} = 1.99$, $p > 0.05$; total: $F_{(1,53)} = 0.01$; $p > 0.05$).

We then examined the expression of the GABA_A α 4 subunit, which is a component of extrasynaptic receptors expressed on MSN but is also expressed by interneurons (see Introduction). There was no alteration in total (WD2: $t_{22} = 0.76$, $p > 0.05$; WD25: $t_{19} = 0.84$, $p > 0.05$; WD48: $t_{19} = 0.64$, $p > 0.05$) or surface (WD2: $t_{22} = 0.11$, $p > 0.05$; WD25: $t_{19} = 0.77$, $p > 0.05$; WD48: $t_{19} = 0.75$, $p > 0.05$) expression of the GABA_A α 4 subunit at any of the withdrawal time-points examined (Fig. 2). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times (surface: $F_{(1,57)} = 0.13$, $p > 0.05$; total: $F_{(1,55)} = 0.27$, $p > 0.05$).

Finally, we examined the expression of the GABA_A α 1 subunit, which is preferentially expressed on interneurons (see Introduction). There was no alteration in total (WD2: $t_{20} = 0.34$, $p > 0.05$; WD25: $t_{20} = 0.67$, $p > 0.05$; WD48: $t_{19} = 0.30$, $p > 0.05$) or surface (WD2: $t_{22} = 0.79$, $p > 0.05$; WD25: $t_{16} = 0.31$, $p > 0.05$; WD48: $t_{19} = 0.32$, $p > 0.05$) expression of the GABA_A α 1 subunit at any of the withdrawal time-points examined (Fig. 3). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times (surface: $F_{(1,54)} = 0.06$, $p > 0.05$; total: $F_{(1,56)} = 0.21$, $p > 0.05$).

3. Discussion

The state of drug addiction results from complex neuroplasticity including homeostatic cascades that can both promote and oppose drug craving (Koob & Volkow, 2010; Huang et al., 2011). We chose to investigate potential plasticity of GABA transmission using the ‘incubation of cocaine craving’ model. This model is relevant to a common pattern of human drug abuse in which users undergo a period of heavy drug-taking that is followed by a period of abstinence (imposed by hospitalization or incarceration); during abstinence, incubation of cue-induced cocaine craving may occur and increase vulnerability to relapse (Reichel & Bevins, 2009; Pickens et al., 2011). In fact, incubation of craving has been observed in clinical studies of humans addicted to nicotine (Bedi et al., 2009), methamphetamine (Wang et al., 2013) and alcohol (Li et al., 2015).

Using an extended-access cocaine self-administration regimen leading to incubation of craving, our lab previously discovered that CP-AMPA levels increase in excitatory

synapses onto NAc core MSN after ~1 month of abstinence and thereafter mediate the expression of incubated cocaine craving (Conrad et al., 2008; Loweth et al., 2014). Since CP-AMPA receptors exhibit higher conductance compared to Ca^{2+} -impermeable AMPARs, it is expected that the NAc response to afferent glutamatergic excitation will be enhanced (for review, see: Isaac et al., 2007; Lee et al., 2012). In fact, we found a significant increase in the responsiveness of NAc core MSN to synaptic stimulation after withdrawal periods sufficient to lead to increased synaptic levels of CP-AMPA receptors (Purgianto et al., 2013). Other work, mainly focused on glutamate inputs to NAc shell MSN, has demonstrated pathway-specific plasticity after withdrawal from cocaine self-administration (Suska et al., 2013; Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014; Terrier et al., 2015).

Based on the observations described above, it is reasonable to ask whether there is a homeostatic response to enhanced glutamate drive in the NAc of “incubated rats”, perhaps mediated by alteration of the GABAergic microcircuit. We focused on NAc core, where a causal relationship between elevated CP-AMPA receptor levels and incubation of craving is well established (Conrad et al., 2008; Loweth et al., 2014). Other evidence also implicates NAc core MSN in the incubation of cocaine craving (Hollander et al., 2005; Hollander et al., 2007; Guillem et al., 2014). We began by studying two GABA_A α receptor subunits ($\alpha 2$ and $\alpha 4$) that are expressed by NAc MSN (Schwarzer et al., 2001; Boyes & Bolam 2007; Maguire et al., 2014).

We discovered that there was an elevation of GABA_A $\alpha 2$ subunit surface expression on WD2 but not WD25 or WD48, suggesting a transient increase in inhibitory tone onto MSN early in withdrawal. This could contribute to low levels of cue-induced cocaine seeking in early withdrawal. Our results on GABA_A $\alpha 2$ subunit expression may also be suggestive of a failure of homeostasis, both at long withdrawal times (when we failed to observe an increase in GABA_A receptor surface expression that might compensate for increased AMPA transmission) and at short withdrawal times as well. Thus, on WD1, biochemical evidence indicates that the responsiveness of NAc core MSN to excitatory inputs may be decreased due to a reduction in cell surface GluA1 levels (Conrad et al., 2008). The increase in GABA_A $\alpha 2$ surface expression observed on WD2 would serve to further reduce NAc responsiveness. Another potential interpretation is that the up-regulation of $\alpha 2$ expression is a protective mechanism. Dixon et al. (2014) observed that the level of cocaine intake in wild-type animals decreased over the course of 10 self-administration sessions, while the level of intake of global GABA_A $\alpha 2$ knock-out animals stayed constant. Extrapolating from these behavioral data, our observed increase in $\alpha 2$ surface expression may represent a compensatory mechanism that limits drug intake. However, behavioral data from knock-out animals may not be directly comparable to data from wild-type animals, since genetic deletion of the subunit may lead to compensatory mechanisms not seen on WT animals.

Our findings indicate that the surface and total expression of the GABA_A $\alpha 4$ subunit was not altered during withdrawal from extended-access cocaine self-administration. No prior studies have examined the effect of cocaine on $\alpha 4$ subunit expression in the NAc, although Heiman et al. (2008) found a significant increase in GABA_A $\alpha 4$ gene expression in dorsal striatum soon after (~4h) the last of fifteen of daily cocaine injections. Functionally, $\alpha 4$ -containing GABA_A receptors exert a tonic inhibitory influence onto MSN of the NAc and

appear to be important in suppressing the rewarding effect of cocaine, since pharmacological stimulation of this receptor blocks cocaine-induced enhancement of CPP while their genetic deletion enhances cocaine-induced CPP (Maguire et al., 2014). Two microdialysis studies found that NAc levels of extracellular GABA, which is the most likely source for GABA_A α 4 activation, are altered after discontinuing cocaine exposure (Xi et al., 2003 – core/shell placements; Wydra et al., 2013 – shell) and adaptations in the expression of GABA_A α 4 might therefore be expected. However, depending on the regimen, different changes in GABA levels were observed (Xi et al., 2003; Wydra et al., 2013). Our cocaine regimen differs in many respects from these prior studies, making it difficult to predict whether any alterations in extracellular GABA levels would be expected under our experimental conditions.

We also did not find any alteration in GABA_A α 1 subunit surface expression after our cocaine self-administration regimen. In the NAc, it has been suggested that this type of receptor is mainly expressed on interneurons (Schwarzer et al., 2001). The most likely pre-synaptic GABA sources to NAc interneurons are either MSN axon collaterals or projection neurons from globus pallidus (Tepper et al., 2010). Although the amount of GABA neurotransmitter released from these sources may be altered by cocaine exposure (Xi et al., 2003; Wydra et al., 2013), our study suggests GABA receptors expressed on interneurons in the NAc do not undergo an adaptation in response to such changes. We have also failed to find evidence for altered activity of GABAergic interneurons after >48 days of withdrawal from the same cocaine regimen used herein (Purgianto et al., 2014). No study has been done to evaluate the functional role of the GABA_A α 1 subunit in the context of cocaine self-administration.

While our study focused on tissue obtained primarily from the NAc core, a recent study of NAc shell found evidence for dynamic changes in the balance of GABA and glutamate transmission during withdrawal from limited-access cocaine self-administration (Otaka et al., 2013). On WD1, they observed a decrease in the relative weight of excitatory to inhibitory synaptic inputs to NAc shell MSN (defined operationally as the ratio of the peak amplitude of EPSCs divided by the peak amplitude of IPSCs). If the decrease in GABA_A α 2 subunit surface expression that we detected in core on WD2 also occurs in shell, this could help explain their WD1 results. On WD21, Otaka et al. (2013) found that the excitatory/inhibitory ratio in NAc shell was increased, probably reflecting a combination of decreased amplitude of GABA_AR mIPSCs and enhanced AMPAR transmission detected at this withdrawal time (Otaka et al., 2013). It will be important to conduct similar recording studies in NAc core MSN.

Overall, two main conclusions can be drawn from our study. First, surface expression of the GABA_A receptor subunits α 1, α 2, and α 4 is not altered during the late withdrawal period when we have previously demonstrated enhanced AMPAR transmission in the NAc and elevated cue-induced cocaine craving. These results suggest the absence of homeostatic changes involving GABA transmission. Therefore, it may be of interest to investigate whether enhancement of GABA transmission during abstinence can attenuate cocaine craving and prevent relapse by offsetting increased AMPAR transmission. Many studies have shown that the GABA_B agonist baclofen attenuates cocaine-related behaviors in animal

models of addiction, including tests of drug seeking during abstinence (Filip et al., 2015), and some results of clinical trials suggest that baclofen may be of greater benefit during abstinence as compared to during active cocaine use (Shoptaw et al., 2003; Kahn et al., 2009). Second, our observation of a transient elevation in $\alpha 2$ subunit surface expression in early withdrawal (WD2), which returned to normal levels by WD25, could help explain low levels of cocaine seeking in early withdrawal, although its functional significance remains to be examined. More generally, it may serve as a component of maladaptive processes that pave the way for incubation of cocaine craving and accompanying adaptations observed later in withdrawal.

4. Experimental Procedures

4.1. Jugular catheterization surgery and self-administration

Our procedures for jugular catheterization surgery and cocaine self-administration have been described in detail previously (Conrad et al., 2008). Briefly, adult male Sprague-Dawley rats (250–275 g on arrival; Harlan, Indianapolis, IN) were housed singly in standard rat cages (lights on at 1900 hours, off at 0700 hours; food and water available *ad libitum*). After ~1 week to acclimate to the colony, rats were surgically implanted with a jugular catheter (PlasticsOne, Roanoke, VA) under ketamine-xylazine anesthesia (80–10mg/kg, i.p., respectively) to enable intravenous drug self-administration. Rats received the analgesic flunixin meglumine (2mg/kg, s.c; Henry Schein, Melville, NY) before surgical procedures. Immediately after surgery and each day during the recovery period (5–7 days), intravenous antibiotic was administered (Cefazolin; 100mg/ml, 0.15ml; Moore Medical, Farmington, CT) and the catheters were flushed with sterile saline solution to ensure patency. After this recovery period, rats began self-administration training in operant chambers (MED Associates, St Albans, VT). All self-administration sessions were conducted during the dark cycle. Nose-poking in the inactive hole had no consequences, whereas nose-poking in the active hole delivered an infusion of saline or cocaine (0.5 mg/kg in a 100 μ l/kg volume over 3 s), paired with a 20 s light cue inside the nose-poke hole. All procedures were performed in accordance with the USPHS Guide for Care and Use of Laboratory Animals and EC Directive 86/609/EEC, and were approved by the Rosalind Franklin University of Medicine and Sciences Institutional Animal Care and Use Committee.

4.2 Biotinylation

All samples were collected and processed as described previously (Ferrario et al., 2011). Briefly, animals were decapitated and brains were rapidly removed. The NAc (mainly core, but sometimes including a portion of lateral shell; see diagram in McCutcheon et al., 2011) was punched from a 2 mm coronal section obtained using a brain matrix. Bilateral pieces of NAc tissue from each rat were minced with a scalpel. Tissue was then added to eppendorf tubes containing ice-cold aCSF with 1mM sulfo-NHS-S-S-Biotin (Thermo Scientific, Rockford, IL) and incubated at 4°C with gentle agitation for 30 min. Samples were incubated with 100mM glycine at 4°C for 10 min to quench the reaction. They were then pelleted, re-suspended in ice-cold lysis buffer [25mM HEPES pH 7.4, 500mM NaCl, 2mM EDTA, 20mM NaF, 10mM NaPPi, 1mM PMSF, 0.1% NP-40 (v/v), 1mM NaOV, 1 μ M okadaic acid, 1 μ M microcystin-LF, 1 $\times$ protease inhibitor cocktail (Calbiochem 539131,

EMD Millipore, Billerica, MA)], sonicated, and stored at -80°C . An aliquot of each sample (100 μg) was added to 37.5 μg of high capacity NeutrAvidin agarose beads (Thermo Scientific, Rockford, IL) and incubated overnight at 4°C on an end-over-end rotator. Biotinylated proteins bound to NeutrAvidin beads (bound fraction) were isolated from the nonbiotinylated (unbound) fraction by centrifugation (3000 RPM, 1min) and washed several times with $1\times$ PBS. The biotinylated fraction was then dissolved in Laemmli sample buffer with DTT (100mM) and heated at 97°C for 3 min to release the biotinylated protein from the beads. The samples were then spun at 10,000 RPM for 5 min on a centrifugal filter unit (0.45 μm , UFC30HV00, EMD Millipore, Billerica, MA) to remove the NeutrAvidin beads from the solution. Samples were used for immunoblotting as described below.

4.3 SDS-PAGE and immunoblotting

Samples were heated to 70°C in Laemmli sample treatment buffer and electrophoresed on 4–12% bis-tris gradient gels (Cat# 345-0125; BioRad, Des Plaines, IL) under reducing conditions. Proteins were then transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Using the SNAP-ID 2.0 protein detection system (EMD Millipore, Billerica, MA), PVDF membranes were incubated with blocking solution (0.5% non-fat milk and 1% goat serum in TBS-Tween20 (TBS-T); 10 min), which was followed by incubation with primary antibodies for GABA_A receptor subunits $\alpha 1$ (1:333; 75–136; Neuromab, Davis, CA), $\alpha 2$ (1:333; AB72445; ABCAM, Cambridge, MA), or $\alpha 4$ (1:10; 73–383; Neuromab, Davis, CA) for 30 min. Membranes were washed 4 times with TBS-T, followed by incubation with secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse; 1:3000; Invitrogen, Carlsbad, CA). Membranes were then washed 4 times with TBS-T and immersed in chemiluminescence (ECL) detecting substrate (GE Healthcare, Piscataway, NJ). Images were acquired with an Amersham Imager 600 (GE Healthcare, Piscataway, NJ) and quantified with TotalLab software (TotalLab; Newcastle, UK). Data were excluded if there were problems that interfered with band analysis such as bubbles. A background value was obtained and diffuse densities for bands of interest in each lane were determined. For data obtained from starting material, diffuse densities were normalized to either total protein in the lane as determined by Ponceau staining (P7170-1L; Sigma-Aldrich, St. Louis, MO) or a loading control (GAPDH; CB1001; EMD Millipore, Billerica, MA). Controls were performed to verify that intracellular proteins such as tyrosine hydroxylase were not detected in the bound fraction.

4.4 Statistical analysis

Results are expressed as mean $\pm$ SEM. Two-tail unpaired t-tests were used to assess group differences (cocaine vs. saline) in GABA_A subunit expression at each withdrawal time-point. Two-way ANOVA was used to analyze differences (cocaine vs. saline) through the course of withdrawal (WD2, WD25, and WD48).

Acknowledgments

This work was supported by USPHS grants DA009621 and DA029099 to MEW and K99 DA038110 to JAL.

References

- Bedi G, Preston KL, Epstein DH, Heishman SJ, Marrone GF, Shaham Y, de Wit H. Incubation of cue-induced cigarette craving during abstinence in human smokers. *Biol. Psychiatry*. 2011; 69(7):708–711. [PubMed: 20817135]
- Bennett BD, Bolam JP. Synaptic input and output of parvalbumin-immunoreactive neurons in the neostriatum of the rat. *Neuroscience*. 1994; 62(3):707–719. [PubMed: 7870301]
- Boyes J, Bolam JP. Localization of GABA receptors in the basal ganglia. *Prog. Brain Res*. 2007; 160:229–243. [PubMed: 17499117]
- Chen Q, Lee TH, Wetsel WC, Sun Q-A, Liu Y, Davidson C, Xiong X, Ellinwood EH, Zhang X. Reversal of cocaine sensitization-associated changes in GAD67 and GABA_A receptor $\alpha 2$ subunit expression and PKC γ activity. *Biochem. Biophys. Res. Commun*. 2007; 356(3):733–738. [PubMed: 17382295]
- Conrad KL, Tseng KY, Uejima JL, Reimers JM, Heng L-J, Shaham Y, Marinelli M, Wolf ME. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature*. 2008; 454:118–121. [PubMed: 18500330]
- Dixon CI, Morris HV, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, Vallada H, Guindalini C, Laranjeira R, Messas G, Rosahl TW, Atack JR, Peden DR, Belleli D, Lambert JJ, King SL, Schumann G, Stephens DN. Cocaine effects on mouse incentive-learning and human addiction are linked to $\alpha 2$ subunit-containing GABA_A receptors. *Proc. Natl. Acad. Sci. USA*. 2010; 107(5):2289–2294. [PubMed: 20133874]
- Dixon CI, Halbout B, King SL, Stephens DN. Deletion of the GABA_A $\alpha 2$ subunit does not alter self-administration of cocaine or reinstatement of cocaine seeking. *Psychopharmacology*. 2014; 231:2695–2703. [PubMed: 24481569]
- Ferrario CR, Loweth JA, Milovanovic M, Ford KA, Galiñanes GL, Heng L-J, Tseng KY, Wolf ME. Alterations in AMPA receptor subunits and TARPs in the rat nucleus accumbens related to the formation of Ca²⁺-permeable AMPA receptors during the incubation of cocaine craving. *Neuropharmacology*. 2011; 61:1141–1151. [PubMed: 21276808]
- Filip M, Frankowska M, Sadakierska-Chudy A, Suder A, Szumiec L, Mierzejewski P, Bienkowski P, Przeglasiński E, Cryan JF. GABA_B receptors as a therapeutic strategy in substance use disorders: Focus on positive allosteric modulators. *Neuropharmacol*. 2015; 88:36–47.
- Gittis AH, Nelson AB, Thwin MT, Palop JJ, Kreitzer AC. Distinct roles of GABAergic interneurons in the regulation of striatal output pathway. *J. Neurosci*. 2010; 30(6):2223–2234. [PubMed: 20147549]
- Groenewegen HJ, Wright CI, Beijer AVJ, Voorn P. Convergence and segregation of ventral striatal inputs and outputs. *Ann. N. Y. Acad. Sci*. 1999; 877:49–63. [PubMed: 10415642]
- Gruber AJ, Powell EM, O'Donnell P. Cortically activated interneurons shape spatial aspects of cortico-accumbens processing. *J. Neurophysiol*. 2009; 101(4):1876–1882. [PubMed: 19176610]
- Guillem K, Ahmed SH, Peoples LL. Escalation of cocaine intake and incubation of cocaine seeking are correlated with dissociable neuronal processes in different accumbens subregions. *Biol. Psychiat*. 2014; 76(1):31–39. [PubMed: 24120118]
- Heiman M, Schaefer A, Gong S, Peterson JD, Day M, Ramsey KE, Suarez-Farinas M, Schwarz C, Stephan DA, Surmeier DJ, Greengard P, Heintz N. A translational profiling approach for the molecular characterization of CNS cell types. *Cell*. 2008; 135(4):738–748. [PubMed: 19013281]
- Hollander JA, Carelli RM. Abstinence from cocaine self-administration heightens neural encoding of goal-directed behaviors in the accumbens. *Neuropsychopharmacol*. 2005; 30(8):1464–1474.
- Hollander JA, Carelli RM. Cocaine-associated stimuli increase cocaine seeking and activate accumbens core neurons after abstinence. *J. Neurosci*. 2007; 27(13):3535–3539. [PubMed: 17392469]
- Huang YH, Schlüter O, Dong Y. Cocaine-induced homeostatic regulation and dysregulation of nucleus accumbens neurons. *Behav. Brain Res*. 2011; 216(1):9–18. [PubMed: 20708038]
- Hussain Z, Johnson LR, Totterdell S. A light and electron microscopic study of NADPH-diaphorase-, calretinin-, and parvalbumin-containing neurons in the rat nucleus accumbens. *J. Chem. Neuroanat*. 1996; 10:19–39. [PubMed: 8703362]

- Isaac JT, Ashby MC, McBain CJ. The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. *Neuron*. 2007; 54(6):859–871. [PubMed: 17582328]
- Janssen MJ, Yasuda RP, Vicini S. GABA_A receptor $\beta 3$ subunit expression regulates tonic current in developing striatopallidal medium spiny neurons. *Front. Cell Neurosci*. 2011; 5:15. [PubMed: 21847370]
- Kahn R, Biswas K, Childress AR, Shoptaw S, Fudala PJ, Gorgon L, Montoya I, Collins J, McSherry F, Li SH, Chiang N, Alathari H, Watson D, Liberto J, Beresford T, Stock C, Wallace C, Gruber V, Elkashef A. Multi-center trial of baclofen for abstinence initiation in severe cocaine-dependent individuals. *Drug Alcohol Depend*. 2009; 103(1–2):59–64. [PubMed: 19414226]
- Kelley AE. Functional specificity of ventral striatal compartments in appetitive behaviors. *Ann. N. Y. Acad. Sci*. 1999; 877:71–90. [PubMed: 10415644]
- Koob GF, Volkow ND. Neurocircuitry of addiction. *Neuropsychopharmacology*. 2010; 35(1):217–238. [PubMed: 19710631]
- Koos T, Tepper JM. Inhibitory control of neostriatal projection neurons by GABAergic interneurons. *Nat. Neurosci*. 1999; 2(5):467–472. [PubMed: 10321252]
- Koos T, Tepper JM, Wilson CJ. Comparison of IPSCs evoked by spiny and fast-spiking neurons in the neostriatum. *J. Neurosci*. 2004; 24(36):7916–7922. [PubMed: 15356204]
- Lee BR, Ma YY, Huang YH, Wang X, Otaka M, Ishikawa M, Neumann PA, Graziane NM, Brown TE, Suska A, Guo C, Lobo MK, Sesack SR, Wolf ME, Nestler EJ, Shaham Y, Schluter OM, Dong Y. Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving. *Nat. Neurosci*. 2013; 16(11):1644–1651. [PubMed: 24077564]
- Lee HK. Ca²⁺-permeable AMPA receptors in homeostatic synaptic plasticity. *Front. Molec. Neurosci*. 2012; 5:17. [PubMed: 22347846]
- Li P, Wu P, Xin X, Fan YL, Wang GB, Wang F, Ma MY, Xue MM, Luo YX, Yang FD, Bao YP, Shi J, Sun HQ, Lu L. Incubation of alcohol craving during abstinence in patients with alcohol dependence. *Addict Biol*. 2015; 20(3):513–522. [PubMed: 24698092]
- Loweth JA, Scheyer AF, Milovanovic M, LaCrosse AL, Flores-Barrera E, Werner CT, Li X, Ford KA, Le T, Olive MF, Szumlinski KK, Tseng KY, Wolf ME. Synaptic depression via mGluR1 positive allosteric modulation suppresses cue-induced cocaine craving. *Nat. Neurosci*. 2014; 17(1):73–80. [PubMed: 24270186]
- Lu L, Grimm JW, Dempsey J, Shaham Y. Incubation of cocaine craving after withdrawal: a review of preclinical data. *Neuropharmacology*. 2004; 47(Suppl. 1):214–226. [PubMed: 15464139]
- Ma YY, Lee BR, Wang X, Guo C, Liu L, Cui R, Lan Y, Balcita-Pedicino JJ, Wolf ME, Sesack SR, Shaham Y, Schluter OM, Huang YH, Dong Y. Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projection. *Neuron*. 2014; 83(6):1453–1467. [PubMed: 25199705]
- Maguire EP, Macpherson T, Swinny JD, Dixon CI, Herd MB, Belelli D, Stephens DN, King SL, Lambert JJ. Tonic inhibition of accumbal spiny neurons by extrasynaptic $\alpha 4\beta \delta$ GABA_A receptors modulates the action of psychostimulants. *J. Neurosci*. 2014; 34(3):823–838. [PubMed: 24431441]
- Mameli M, Halbout B, Creton C, Engblom D, Parkitna JR, Spanagel R, Luscher C. Cocaine-evoked synaptic plasticity: persistence in the VTA triggers adaptations in the NAc. *Nat. Neurosci*. 2009; 12(8):1036–1041. [PubMed: 19597494]
- McCutcheon JE, Wang X, Tseng KY, Wolf ME, Marinelli M. Calcium-permeable AMPA receptors are present in nucleus accumbens synapses after long withdrawal from cocaine self-administration but not experimenter-administered cocaine. *J. Neurosci*. 2011; 31:5737–5743. [PubMed: 21490215]
- Meredith GE. The synaptic framework for chemical signaling in nucleus accumbens. *Ann. N. Y. Acad. Sci*. 1999; 877:140–156. [PubMed: 10415648]
- Meredith GE, Baldo BA, Andrezejewski ME, Kelley AE. The structural basis for mapping behavior onto the ventral striatum and its subdivisions. *Brain Struct. Funct*. 2008; 213(1–2):17–27. [PubMed: 18256852]
- Morris HV, Dawson GR, Reynolds DS, Atack JR, Rosahl TW, Stephens DN. Alpha2-containing GABA_A receptors are involved in mediating stimulant effects of cocaine. *Pharmacol. Biochem. Behav*. 2008; 90:9–18. [PubMed: 18358520]

- Olsen RW, Sieghart W. International union of pharmacology. LXX. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev.* 2008; 60:243–260. [PubMed: 18790874]
- Olsen RW, Sieghart W. GABA_A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology.* 2009; 56:141–148. [PubMed: 18760291]
- Otaka M, Ishikawa M, Lee BR, Liu L, Neumann PA, Cui R, Huang YH, Schlüter OM, Dong Y. Exposure to cocaine regulates inhibitory synaptic transmission in the nucleus accumbens. *J. Neurosci.* 2013; 33(16):6753–6758. [PubMed: 23595733]
- Pascoli V, Terrier J, Espallergues J, Valjent E, O'Connor EC, Luscher C. Contrasting forms of cocaine-evoked plasticity control components of relapse. *Nature.* 2014; 509(7501):459–464. [PubMed: 24848058]
- Pennartz CM, Kitai ST. Hippocampal inputs to identified neurons in an in vitro slice preparation of the rat nucleus accumbens: evidence for feed-forward inhibition. *J. Neurosci.* 1991; 11(9):2838–2847. [PubMed: 1679123]
- Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y. Neurobiology of the incubation of drug craving. *Trends Neurosci.* 2011; 34(8):411–420. [PubMed: 21764143]
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience.* 2000; 101(4):815–850. [PubMed: 11113332]
- Purgianto A, Scheyer AF, Loweth JA, Ford KA, Tseng KY, Wolf ME. Different adaptations in AMPA receptor transmission in the nucleus accumbens after short vs long access cocaine self-administration regimens. *Neuropsychopharmacology.* 2013; 38:1789–1797. [PubMed: 23546386]
- Purgianto A, Miao J, Milovanovic M, Wolf ME. Effects of extended access cocaine self-administration on inhibitory neurotransmission in the nucleus accumbens. *Soc. Neurosci. Abstr.* 2014; 40:811.19.
- Reichel CM, Bevins RA. Forced abstinence model of relapse to study pharmacological treatments of substance abuse disorder. *Curr. Drug Abuse Rev.* 2009; 2(2):184–194. [PubMed: 19630748]
- Santhakumar V, Jones RT, Mody I. Developmental regulation and neuroprotective effects of striatal tonic GABA_A currents. *Neuroscience.* 2010; 167(3):644–655. [PubMed: 20206233]
- Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W, Sperk G. Distribution of the major gamma-aminobutyric acid_A receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. *J. Comp. Neurol.* 2001; 433(4):526–549. [PubMed: 11304716]
- Sesack SR, Grace AA. Cortico-basal ganglia reward network: microcircuitry. *Neuropsychopharmacology.* 2010; 35(1):27–47. [PubMed: 19675534]
- Shoptaw S, Yang X, Rotherham-Fuller EJ, Hsieh YC, Kintaudi PC, Charuvastra VC, Ling W. Randomized placebo-controlled trial of baclofen for cocaine dependence: preliminary effect for individuals with chronic patterns of cocaine use. *J. Clin. Psychiatry.* 2003; 64(12):1440–1448. [PubMed: 14728105]
- Silberberg G, Bolam JP. Local and afferent synaptic pathways in the striatal microcircuitry. *Curr. Opin. Neurobiol.* 2015; 33:182–187. [PubMed: 26051382]
- Suska A, Lee BR, Huang YH, Dong Y, Schlüter OM. Selective presynaptic enhancement of the prefrontal cortex to nucleus accumbens pathway by cocaine. *Proc. Natl. Acad. Sci. USA.* 2013; 110(2):713–718. [PubMed: 23267100]
- Taverna S, van Dongen YC, Groenewegen HJ, Pennartz CM. Direct physiological evidence for synaptic connectivity between medium-sized spiny neurons in rat nucleus accumbens in situ. *J. Neurophysiol.* 2004; 91(3):1111–1121. [PubMed: 14573550]
- Taverna S, Canciani B, Pennartz CM. Membrane properties and synaptic connectivity of fast-spiking interneurons in rat ventral striatum. *Brain Res.* 2007; 1152:49–56. [PubMed: 17459351]
- Tepper JM, Koos T, Wilson CJ. GABAergic microcircuit in the neostriatum. *Trends Neurosci.* 2004; 27(11):662–669. [PubMed: 15474166]
- Tepper JM, Tecuapetla F, Koos T, Ibanez-Sandoval O. Heterogeneity and diversity of striatal GABAergic interneurons. *Front. Neuroanat.* 2010; 4:150.
- Terrier J, Lüscher C, Pascoli V. Cell-type specific insertion of GluA2-lacking AMPARs with cocaine exposure leading to sensitization, cue-induced seeking and incubation of craving. *Neuropsychopharmacology.* 2015 Nov 20. 2015 [Epub ahead of print].

- Tunstall MJ, Oorschot DE, Kean A, Wickens JR. Inhibitory interactions between spiny projection neurons in the rat striatum. *J. Neurophysiol.* 2002; 88(3):1263–1269. [PubMed: 12205147]
- Wang G, Shi J, Chen N, Xu L, Li J, Li P, Sun Y, Lu L. Effects of length of abstinence on decision-making and craving in methamphetamine abusers. *PLoS One.* 2013; 8(7):e68791. [PubMed: 23894345]
- Wilson CJ. GABAergic inhibition in the neostriatum. *Prog. Brain Res.* 2007; 160:91–110. [PubMed: 17499110]
- Wolf ME, Ferrario CR. AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. *Neurosci. Biobehav. Rev.* 2010; 35(2):185–211. [PubMed: 20109488]
- Wolf ME, Tseng KY. Calcium-permeable AMPA receptors in the VTA and nucleus accumbens after cocaine exposure: when, how, and why? *Front. Mol. Neuro.* 2012; 5:75.
- Wolf ME. Synaptic mechanisms underlying persistent cocaine craving. *Nat. Rev. Neurosci.* 2016 in press.
- Wydra K, Golembiowska K, Zaniewska M, Kaminska K, Ferraro L, Fuxe K, Filip M. Accumbal and pallidal dopamine, glutamate, and GABA overflow during cocaine self-administration and its extinction in rats. *Addict. Biol.* 2013; 18(2):307–324. [PubMed: 23311632]
- Xi ZX, Ramamoorthy S, Shen H, Lake R, Samuvel DJ, Kalivas PW. GABA transmission in the nucleus accumbens is altered after withdrawal from repeated cocaine. *J. Neurosci.* 2003; 23(8): 3498–3505. [PubMed: 12716959]

Highlights

- Cocaine craving incubates during withdrawal from cocaine self-administration (SA).
- GABA is critical for regulating NAc output but its role in incubation is unknown.
- GABA_AR α 1, α 2, and α 4 subunits were measured during withdrawal from cocaine SA.
- In early withdrawal (day 2), α 2 surface expression was increased in NAc core.
- No alterations in GABA_AR subunits were found in late withdrawal (25 or 48 days).

Figure 1.

Effect of extended-access cocaine administration and different periods of withdrawal on the expression of GABA_A α2 receptor subunits in the NAc. (a,b) On WD2 after discontinuing cocaine self-administration, total expression of GABA_A α2 subunits was unchanged (t-test, $p > 0.05$), while there was a significant increase in surface expression of this subunit (t-test, $*p < 0.05$). (c,d) On WD25, total expression of GABA_A α2 subunits remained unchanged while surface expression of the subunit returned to a level comparable to saline controls (t-tests, $p > 0.05$). (e,f) On WD48, total and cell surface levels of GABA_A α2 subunits did not

differ between cocaine and saline groups (t-tests, $p > 0.05$). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times ($p > 0.05$). Representative blots show data from 4 different animals: 2 cocaine animals and 2 saline animals. Numbers within the bars indicate the number of rats in each experimental group.

Figure 2.

Effect of extended access cocaine administration and different periods of withdrawal on the expression of GABA_A α4 subunits in the NAc. Saline and cocaine rats did not differ in either surface or total expression of GABA_A α4 subunits on WD2 (a,b), WD25 (c,d), or WD48 (e,f) (t-tests, $p > 0.05$). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times ($p > 0.05$). Representative blots show data from 4 different animals: 2 cocaine animals and 2 saline animals. Numbers within the bars indicate the number of rats in each experimental group.

Figure 3.

Effect of extended access cocaine administration and different periods of withdrawal on the expression of GABA_A α1 subunits in the NAc. Saline and cocaine rats did not differ in either surface or total expression of GABA_A α4 subunits on WD2 (a,b), WD25 (c,d), or WD48 (e,f) (t-tests, $p > 0.05$). Two-way ANOVA revealed no significant difference between cocaine and saline groups over different withdrawal times ($p > 0.05$). Representative blots show data from 4 different animals: 2 cocaine animals and 2 saline animals. Vertical line in the surface blot in the middle panel indicates that lanes were not adjacent in the original

immunoblot. Numbers within the bars indicate the number of rats in each experimental group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript